6 Methods

6.1 Molecular biology techniques

6.1.1 Polymerase chain reaction (PCR)

Double stranded DNA was amplified from a template by polymerase chain reactions (PCRs) using 20 to 30 bp long oligonucleotides as primers. Primers were designed so that specific restrictions sites were introduced at the 5' and 3' ends of the amplified PCR products. All PCR reactions were set up according to the following scheme (Table 6.1) and the standard PCR program is given in Table 6.2. All synthetic oligonucleotides used are listed under 5.7.

	amount
template DNA	100-200 ng
10x ThermoPol buffer	5µl (1x)
dNTPs (10mM)	10 nmol each
5'primer (50µM)	25 pmol
3'primer (50µM)	25 pmol
Deep Vent (2U/µl)	1 U
H_2O	to 50µl

Table 6.1: Standard PCR reaction

Segment	process	temp. [°C]	Time [min:sec]
1	melting	95	5:00
2	melting	95	0:30
	annealing	58	0:30
	extension	72	1:00/1000bp
25 cycles			
3	extension	72	10min

Table 6.2: Standard PCR program

6.1.2 PCR-based mutagenesis

For the introduction of point mutations, primers with the changed sequence were designed so that at least 10 bp flanked the mutation site(s). These primers were used in a first PCR reaction according to the standard PCR protocol given in chapter 6.1.1 together with either the 5' or 3' end primer to create a so-called Megaprimer.

	amount
template DNA	5 ng
10x ThermoPol buffer	5 µl (1x)
dNTPs (10mM)	10 nmol each
MegaPrimer	100-200 ng
5´or 3´primer (50µM)	25 pmol
Deep Vent Polymerase (2U/µl)	1 U
H_20	to 50 µl

Table 6.3: Second PCR reaction for PCR-based mutagenesis

segment	process	Temp.	Time [min:sec]
1	melting	95	5:00
2	melting	95	0:30
	annealing	55	1:00
	extension	72	1:00/1000bp
30 cycles			
3	extension	72	10min

Table 6.4: PCR program of second PCR reaction for PCR-based mutagenesis

The PCR reaction was loaded on an 1% agarose gel, the Megaprimer was cut and purified with the Gelextraction Kit (Qiagen) according to manufacturer's instructions (see 6.1.4). The purified Megaprimer was then used in a second PCR reaction with either the 3' or 5' end primer to create a full length PCR product containing the desired mutations and specific restriction site at each end. Second PCRs for mutagenesis were performed according to Tables 6.3 and 6.4.

6.1.3 PCR purification and restriction digests

PCRs were purified using the PCR purification Kit from Qiagen according to manufacturer's instructions. Subsequently restriction digests of PCR products and vectors were set up as double digests according to the following standard protocol (Table 6.5) and incubated at 37 °C for 2 –12 hours.

	amount
PCR reaction/vector	50 µl/ 5µg
10x reaction buffer	5µl (1x)
100x BSA	0.5 µl (1x)
restriction enzyme 1 (10-20 U/µl)	1-2 µl (10-20 U)
restriction enzyme 2 (10-20 U/µl)	1-2 µl (10-20 U)
H_2O	to 50 µl

Table 6.5: Standard protocol for restriction digests

6.1.4 Agarose gel electrophoresis and gel extraction

Agarose gel electrophoresis was used to separate linearized DNA fragments according to size. 1g agarose was melted in 100 ml 1x TBE buffer and supplemented with 0,2 µg/ml ethidium bromide to pour 1% horizontal agarose gels. Gels were run in TBE buffer at up to 100V.
For purification of digested PCR reactions or vectors the DNA bands were cut with a razor blade and processed using the Gelextraction Kit (Qiagen) according to manufacturer's instructions. Gel purified vectors and PCR products were used for ligation.

6.1.5 Ligation

For ligation reactions approximately 100 ng vector was incubated with a 3 to 10-fold excess of insert DNA and 3 U T4 DNA ligase in 1x ligation buffer for 2 to 16 hours at room temperature.

6.1.6 Preparation of competent *E. coli*

Competent *E. coli* DH5α were prepared according to the calcium chloride method. 250 ml SOB medium were inoculated with 10 ml of an overnight DH5α culture (LB with 10 µg/ml

tetracycline) and bacteria were grown shaking at 20 °C to an OD_{600nm} of 0.6. Bacteria were chilled on ice for 10 min, pelleted and resuspended in 80 ml ice cold TB buffer. After another round of incubation on ice for 10 min and centrifugation, DH5α were resuspended in 20 ml cold TB supplemented with DMSO to a final concentration of 7% (v/v). 200 µl aliquots of competent DH5α were snap frozen in liquid nitrogen and stored at –80 °C.

6.1.7 Transformation

One ligation reaction or 100 – 500 ng of plasmid DNA were used for transformation of competent *E. coli* DH5α. Bacteria were thawed on ice and 90 µl were aliquoted into pre-chilled eppendorf tubes. After a 10 min incubation with the DNA on ice, DH5α were heat-shocked at 42° for 90 sec and cooled down on ice for 60 sec. 900 µl of LB medium were added to each sample and cells were grown for 60 min at 37 °C with vigorous shaking. For ligation reactions bacteria were plated on an LB plate containing the appropriate antibiotic for selection. For preparation of larger DNA amounts (Maxi Prep) 500 µl of the transformed bacteria suspension were used to inoculate 150 ml LB medium containing the appropriate antibiotic.

6.1.8 Isolation of plasmid DNA

For isolation of small amounts of plasmid DNA (Mini-Preps) e.g. after ligation to test colonies for successful cloning, 2 mL LB medium were inoculated with a single colony and bacteria were grown overnight by shaking at 37 °C. The next day, bacteria were pelleted, resuspended in 150 µl buffer P1 (25 mM Tris pH 8, 10 mM EDTA, 100 µg/ml RNAse A) and lysed by adding 150 µl buffer P2 (0.2 M NaOH, 1% SDS (w/v)). Neutralization of the lysates was achieved by addition of 150 µl cold buffer P3 (11.5 % (v/v) acetic acid, 5 M potassium acetate), which leads to the precipitation of SDS-protein- and higher molecular DNA- and RNA-complexes, while circular plasmid DNA stays in solution. After centrifugation for 10 min at 14000 rpm the supernatant was mixed with 1 ml cold ethanol (-20 °C). The precipitated DNA was pelleted by centrifugation (14000 rpm, 10 min), washed once with cold 70 % ethanol (-20 °C), air-dried and finally resolved in 20 µl water. If a greater purity of DNA samples was needed (e.g. for sequencing) the resolved DNA was diluted 5-fold with buffer PB (Qiagen) and further purified using the Qiaprep Spin MiniPrep Kit (Qiagen) according to manufacturer's instructions.

For isolation of large amounts of DNA (Maxi-Preps) a 50 ml *E. coli* culture was grown overnight and DNA was isolated using the Qiagen MaxiPrep Kit following the given instructions.

6.1.9 Control restriction digest

To test Mini-Preps for successful ligation, restriction digests were set up according to the following scheme (Table 6.6) and incubated for 1 to 2 hours at 37 °C prior to analysis on an agarose gel.

	amount
Mini-Prep	1 µl
10x reaction buffer	1µl (1x)
100x BSA	0.1 µl (1x)
Restriction enzyme 1 (10-20 U/µl)	0.1 µl (1-2 U)
Restriction enzyme 2 (10-20 U/µl)	0.1 µl (1-2 U)
H_2O	To 10 µl

Table 6.6: Standard protocol for control restriction digests

6.1.10 Sequencing

All cloned constructs were verified by sequencing. For sequencing based on the chain termination method, PCR reactions were set up containing fluorescence labeled didesoxynucleotides. The labeled sequencing PCR products were separated by capillary electrophoresis according to their size and analyzed in a 310 sequencer from Applied Biosystems. Sequencing PCRs were performed according to the following protocol and program (Tables 6.7 and 6.8).

	amount
Plasmid DNA	200-300 ng
BigDye Terminator v1.1	0.5 µl
5x Sequencing buffer	2 µl (1x)
Sequencing primer	5 pmol
H_2O	To 10 µl

Table 6.7: Standard sequencing PCR protocol

Segment	process	Temp. [°C]	Time [min:sec]
1	melting	95	3:00
2	melting	96	0:35
	annealing	50	0:12
	extension	60	4:00
25 cycles			

Table 6.8: Standard program for sequencing PCR

Sequencing PCR products were purified using ethanol precipitation: reactions were mixed with 20 µl H_2O and 10 µl 3M sodium acetate and added to 200 µl ethanol. Samples were centrifuged for 15 min at 14000rpm (room temperature) and pelleted DNA was washed with 250 µl 70 % ethanol. After drying for 10 min in a speed vac at room temperature, DNA was resuspended in 20 µl of a special formamid solution (template suppression reagent, Applied

Biosystems). PCR samples were denatured at 90 °C for 2 min and cooled down on ice prior to capillary electrophoresis.

6.2 Cell culture

6.2.1 Culture and plasmid transfection of HEK293 cells

Culture

HEK293 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % fetal calf serum (FCS), penicillin (100 U/ml) / streptomycin (0.1 mg/ml) and sodium pyruvate (1 mM). Routinely cells were splitted every 2 to 3 days. For that purpose, cells were washed once with 10 ml PBS and incubated with 1ml trypsin/EDTA solution (per 10 cm dish) for 1 to 2 min at 37 °C. Detached cells were washed off by adding 9 ml of complete DMEM medium, which stops the tryptic digestion reaction, diluted as necessary (normally 1:3 to 1:5) and seeded in new culture dishes.

Plasmid transfection

HEK293 cells were transfected using calcium phosphate precipitation. During their growth $CaPO_4$-crystals can embed plasmid DNA, that is present in the solution. Cells are able to take up those crystals and therefore transiently express the desired protein(s). For transfection of HEK293 1.5 x 10^6 cells were plated per 10 cm dish. After 24 hours plasmid transfection was performed: 10 µg total DNA was mixed with 250 µl H_2O and 38 µl 2 M $CaCl_2$ and then added drop wise to 300 µl 2x HBS buffer while vortexing. After incubation at room temperature for 20 min small crystals can be observed under a light microscope and the reaction was carefully trickled onto the cells. HEK293 cells were lysed 24 h after transfection.

6.2.2 Culture and plasmid transfection of Phoenix packaging cells

Culture

Phoenix packaging cells were cultured on 15 cm dishes in DMEM medium supplemented with 10 % FCS, penicillin (100 U/ml) / streptomycin (0.1 mg/ml), glutamine (2 mM) and β-mercaptoethanol (50 µM). Passaging was performed as for HEK293 cells (see 6.2.1).

Plasmid transfection

One 15 cm dish of confluent Phoenix cells was splitted into ten 10 cm dishes and cells were grown over night. Transfection was performed according to a modified calcium phosphate precipitation protocol: 20 µg DNA (per 10-cm dish) was mixed with 50 µl 2.5 M $CaCl_2$ (-20 °C) and water (to 500 µl) and then added drop wise to 500 µl 2x HBS-P buffer while

vortexing. After 15 min incubation at room temperature the mix was carefully trickled onto the cell lawn. Virus was harvested after 48 and 72 hours (see 6.2.5.2).

6.2.3 Culture and plasmid transfection of COS-7 cells

Culture

COS-7 cells were cultivated like HEK293 cells (described in 6.2.1).

Plasmid transfection

Confluent COS-7 cells were diluted 3 fold and plated in 24-well plates (containing cover slips for subsequent immunofluorescence staining). The next day DNA was introduced by lipid mediated transfection using Lipofectamine 2000 reagent. The mechanism of lipid-mediated transfection, also called lipofection, is based on the formation of a lipid/nucleic acid complex, which is able to fuse with the cell membrane and release the nucleic acid inside the cell. 0.5 - 1 µg DNA were diluted in 35 µl OptiMEM medium. 1 µl of Lipofectamine was dissolved in 35 µl OptiMEM medium and added to the diluted DNA. The mixture was incubated for 10 to 15 min at room temperature. DMEM medium was removed from the cell layer and substituted by 200 µl OptiMEM medium before the transfection reactions were added to the cells. Protein expression was analyzed after 24 h.

6.2.4 Jurkat T cells

6.2.4.1 Culture and plasmid transfection

Culture

Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) / streptomycin (0.1 mg/ml) and glutamine (4 mM) at a cell density of 0.5 to 1.5 x 10^6 cells /ml. For continuous passage cells were diluted every 2 to 3 days.

Plasmid transfection

Jurkat T cells were transfected with plasmids using the electroporation technique. During an electric pulse, voltage-controlled pores in the cell membrane of cells in suspension shortly open, so that extracellular plasmid DNA can enter the cell. This leads to transient ectopic expression of the protein(s) of interest. For this purpose 8 x 10^6 Jurkat cells were diluted in 400 µl complete RPMI medium and mixed with the desired plasmid DNA (maximum 30 µg). The cells were then pulsed in an electroporation cuvette (4 mm width) using a Gene Pulser at 200 V and 950 µF. Afterwards cells were resuspended in 10 ml of complete RPMI medium and grown for 48 to 72 h prior to analysis.

6.2.4.2 siRNA transfection

RNA interference (RNAi) is based on a defense mechanism developed by plants and invertebrates against foreign invading genetic elements such as transposons or retroviruses. The trigger for this defense mechanism is double-stranded (ds)RNA, which is broken down by the cellular endonuclease DICER into 21 to 23 bp long fragments commonly called short interfering RNAs (siRNAs). These fragments are then used by RISC (RNA-induced silencing complex) as co-factors to bind and destroy complementary mRNA. Besides, the interferon response gets activated by dsRNA in mammalian cells. This pathway is mainly mediated by the RNA-dependent protein kinase RPK and leads to the general downregulation of protein synthesis and therefore growth arrest and apoptosis. That is why short dsRNAs are normally used in mammalian systems to directly mimic siRNAs. For the knock-down of TRAF6 in Jurkat T cells stealth siRNAs (25 bp, Invitrogen) were used, which are chemically modified to yield greater stability and maximal reduction of the interferon/RPK stress response.

For lipid-mediated siRNA transfection, 800 µl of Jurkat cells were seeded per well of a 24-well plate at a density of 4 x 10^5 cells/ml. 1 µl of a 100 µM siRNA solution was mixed with 100 µl OptiMEM medium in a 96-well. In parallel the Atufect lipid was diluted in OptiMEM medium (1:100), shortly vortexed and 100 µl of this mix were added to each 96-well containing the siRNA. After incubation for 30 min at 37 °C the siRNA/Atufect mix was added to the cells. Transfected Jurkat cells were diluted with 1 ml of fresh RPMI medium after 48 h and analyzed after 72 h. For detection of Malt1 ubiquitination (see 6.4.6) cells of six 24-wells were combined for each time point.

6.2.4.3 Activation

For stimulation of Jurkat T cells, the desired number of cells was spun down at 1000 rpm for 5 min and resuspended in 0.5 to 1 ml fresh complete RPMI medium. After recovery and re-warming for 5 min at 37 °C, cells were stimulated with phorbol-12-myristate-13-acetate (PMA) and ionomycin or antibodies against CD3 and CD28.

Stimulation with PMA/ionomycin:

PMA possesses structural similarity to diacylglycerol (DAG) and can therefore activate PKCθ and thus NF-κB in T cells. Ionomycin induces Ca^{2+} influx from intracellular Ca^{2+} storage compartments and therefore mainly influences the activation of NFAT. For stimulation of Jurkat cells 200 ng/ml PMA and 300 ng/ml ionomycin were added directly to the medium.

Stimulation through CD3/CD28 antibody ligation

Crosslinking of the co-stimulatory receptor CD28 and the T cell receptor (CD3 subunits) by specific primary and secondary antibodies mimics the receptor aggregation, which is under physiological conditions induced by the T cell/APC (antigen presenting cell) contact. This leads to activation of antigen-receptor specific signaling cascades. The following antibodies were used at the given concentrations: anti-hCD3 (IgG_{2a}),1 µg/ml; anti-hCD28 (IgG_1), 5 µg/ml; anti IgG_1, 2.5 µg/ml and anti IgG_{2a}, 2.5 µg/ml. After stimulation cells were placed on ice, washed once with cold PBS and lysed immediately with the indicated buffers.

6.2.5 Primary CD4+ T cells

6.2.5.1 Isolation and culture

Primary CD4+ T cells were isolated from spleen and lymph nodes of wildtype and Malt1 -/- mice [157] through positive selection using mCD4+ Dynabeads and cultured in RPMI supplemented with penicillin (100 U/ml) / streptomycin (0.1 mg/ml), glutamine (2 mM), β-mercaptoethanol (50 µM) and 10% FCS. mCD4+ Dynabeads are magnetic particles coated with anti-CD4 antibodies, with which CD4 expressing cells can be separated from a cell suspension. Spleens and lymph nodes were treated separately: spleens were cut in smaller pieces and homogenized by pressing through a cell strainer, which was dipped into 5ml medium in a 6-well. Lymph nodes were homogenized in the same way. For lysis of erythrocytes the spleenocyte suspension was spun down, resuspended in 5 ml TAC lysis buffer by careful pipetting and incubated on ice for 5 min. Under these conditions the erythrocytes burst first due to their impaired osmotic regulation ability. The lysis reaction was stopped by the addition of 10 ml medium, remaining cells were spun down again and taken up in fresh medium. The lymph node cell suspension was also centrifuged once. Cells were resuspended in fresh medium and then combined with the remaining spleenocytes. The combined cell samples were then spun down and taken up in 3 ml medium containing only 1% FCS. 100 µl of mCD4+ Dynabead suspension per mouse was added to the cells and the mix was incubated for 30 to 60 min rotating slowly at 4 °C. Afterwards, Dynabeads were carefully washed 3 times with 3 ml 1% FCS medium using the Magnetic Particle Concentrator and were finally taken up in 2 ml of complete RPMI medium. Then 50 µl Detachabeads per mouse were added to release CD4 positive (CD4+) T cells from the Dynabeads. The samples were incubated slowly shaking at room temperature for 1 hour and shortly vortexed a couple of times. Afterwards beads were separated from the cells in solution using the Magnetic Particle Concentrator and washed once with complete RPMI medium to

gather remaining sticking cells from the beads. Isolated CD4 positive T cells were diluted to a density of 2 x 10^6 cells/ml and supplemented with mCD3 (0.5 µg/ml) and mCD28 antibodies (1 µg/ml). 1 ml of this cell suspension was then seeded per 6-well of plates, that had before been coated with ICN anti-hamster antibodies: anti-hamster antibody was diluted 33-fold in PBS and 1 ml of this dilution was used to coat one 6-well. Plates were incubated shaking overnight at 4 °C, were washed twice with PBS and sucked dry before seeding of the T cells. Because CD3/28 antibodies bind to the coat of anti-hamster antibodies, T cells settle down and become attached to the plate surface. Cells were cultured on those plates for 48 h prior to retroviral infection.

6.2.5.2 Retroviral infection

For infection of T cells, two rounds of Phoenix cell transfections were performed. For each construct ten 10 cm dishes Phoenix cells were splitted on the first (TF1) and second day (TF2) of the experiment and transfected on the second and third day, respectively (see 6.2.2). For both transfection rounds medium was removed after 24 h and 4.5 ml new medium was added. First virus was harvested from the TF1 phoenix cells after 48 h. The collected medium was centrifuged at 6000 rpm for 12 h to yield a more concentrated virus sample. Fresh medium was added to the TF1 Phoenix cells so that the next day, virus could be harvested again from TF1 Phoenix cells (72 h after transfection) and also from TF2 Phoenix cells (48 h after transfection). For clarity a time schedule of the infection experiment is given in Table 6.9.

Day	Transfection round 1 (TF1)	Transfection round 2 (TF2)	CD4+ T cells
1	split 1 x 15 cm dish (per construct) into 10 x 10 cm dishes		
2	transfection of Phoenix cells	split 1 x 15 cm dish (per construct) into 10 x 10 cm dishes	coating of 6-well plates with anti-hamster antibody
3	medium exchange (add 4.5 ml fresh medium)	transfection of Phoenix cells	isolation of CD4+ T cells from mice, seed in coated wells (add CD3/28 antibodies)
4	harvest virus, spin ON at 6000 rpm, (add 4.5 ml fresh medium to cells)	medium exchange (add 4.5 ml fresh medium)	
5	harvest virus	harvest virus	infection with combined virus samples (7h)
6-7			culture in medium + mIL-2
8			stimulation and analysis

Table 6.9: Time table for retroviral infection of primary CD4+ T cells

All virus samples were combined and filtered through a 0.2 µm membrane, so that 80 - 90 ml of virus were obtained for every construct, which were supplemented with 4 µg/ml polybrene. Medium was sucked of the T cell lawn and infection was carried out by adding 10 ml of virus solution to each 6-well. The 6-well plates were spun at room temperature for 1 h at 2000 rpm to increase infection efficiency. After incubation of the plates at 37 °C for 5 h, the virus solution was removed. T cells were resuspended in complete RPMI medium containing 20 U/ml mIL-2 by carefully washing them off with 1 ml medium per 6-well. Cells were seeded in cell culture flasks at a density of 0.7 -1 x 10^6 cells/ml, diluted as necessary with medium containing fresh mIL-2 after 30 - 40 h and analyzed after 72 hours.

6.2.5.3 Activation

Stimulation with PMA/Ionomycin

Prior to stimulation with PMA/Ionomycin, CD4+ T cells (approximately 1-2 x 10^6 cells/sample) were resuspended in 300 µl medium containing 1.5 µl of Thy1.1-APC antibody (for FACS analysis, see 6.3.1). PMA and Ionomycin were used at a final concentration of 200 ng/ml and added directly to the medium. Stimulation was stopped by adding 300 µl of 4 % para-formaldehyd and cells were subjected to intracellular staining of IκBα (see 6.3.1).

Stimulation with CD3/28 antibodies

Stimulation with CD3/28 antibodies was performed on cell culture plates. For that purpose 6-well plates were coated with anti-hamster antibodies as described in chapter 6.2.5.1. 2 x 10^6 cells per sample were supplemented with 0.5 µg/ml CD3 and 1 µg/ml CD28 antibodies and seeded in one 6-well. Brefeldin A (10 µg/ml f.c.) was added to the cells two hours before analysis to inhibit secretion of IL-2 into the medium. After the indicated stimulation time, cells were washed off the plates and subjected to intracellular staining of IL-2 (see 6.3.1).

6.3 Immunofluoresence

Proteins can be visualized on the surface or inside cells by the use of fluorophore-labeled antibodies. For intracellular staining cell membranes have to be permeabilized to allow the antibodies to penetrate cells (e.g. by the detergent Saponin). Staining can be achieved by a combination of primary and labeled secondary antibodies or by labeled primary antibodies. Antibodies with the fluorophores APC (allophycocyanin, red), FITC (fluorescein, green), Cy3 (cyanine-3, red) and Alexa-Fluor 488 (from Invitrogen, green) were used. Stained proteins can be analyzed by fluorescence microscopy or by fluorescence-activated cell sorting (FACS) using flow cytometry.

6.3.1 Intracellular protein staining in CD4+ T cells for flow cytometric analysis

Phoenix cells were transfected with pMCSV vector constructs which, due to an IRES sequence, lead to the simultaneous expression of the surface marker Thy1.1 (CD90.1) and the different Malt1 constructs. Therefore T cells could be examined for successful infection by staining of Thy1.1 with an APC-labeled antibody. As read-outs for reconstitution of the Malt1 -/- T cells with Malt1 WT or mutant proteins degradation of IκBα and production of IL-2 were measured by intracellular staining of these proteins.

IκBα staining

For detection of IκBα degradation T cells were stimulated with PMA/Ionomycin as described in chapter 6.2.5.3. Thy1.1-APC-labeled antibody was added to the cells prior to stimulation. Stimulation was stopped by adding 300 µl 4% para-formaldehyd (PFA)/PBS for fixation of the cells. After incubation for 15 min at room temperature, cells were washed twice with 3 ml PBS, resuspended in 50 µl 0.5 % Saponin/ 1 % BSA/PBS and incubated for another 10 min at RT for permeabilization. 0.5 µl IκBα antibody (Cell signaling) was then added to each sample for 20 min. Afterwards cells were washed twice with 3 ml PBS and resuspended in 50 µl 0.5 % Saponin/ 1 % BSA/PBS containing secondary antibody (anti-mouse-FITC, fluorescein) at a dilution of 1:300. After a final incubation for 20 min, cells were washed twice with 3 ml PBS before being analyzed by flow cytometry.

IL-2 staining

For analysis of IL-2 production cells were stimulated with plate-bound CD3/28 antibodies as described in chapter 6.2.5.3. After stimulation cells were washed off the plates with 2 ml PBS. The cells were washed once more with 3 ml PBS and then resuspended in 100 µl 1 % FCS/PBS containing 1 µl Thy1.1-APC antibody and incubated for 15 min on ice. To fix the cells 200 µl 4 % PFA/PBS was added and incubated for 10 min at room temperature. After two washing rounds with 3 ml PBS, cells were resuspended in 200 µl 0.5 % Saponin/ 1 % BSA/PBS supplemented with 1 µl FcBlock antibody and incubated for 20 min before 20 µl of a 1:20 dilution of the IL2-FITC antibody was added to each sample. After 30 min of incubation cells were washed twice with 3 ml PBS and analyzed by flow cytometry.

Flag staining

To analyze the expression of FlagMalt1 proteins, 2-3 x 10^6 cells/sample were washed once in PBS and then incubated in 200 µl 1 % FCS/PBS containing 1.5 µl Thy1.1-APC antibody for 15 min on ice. For cell fixation 300 µl 4 % PFA/PBS were added and the cells were incubated for 15 min at room temperature. Cells were washed two times with 3 ml PBS, resuspended in 200 µl 0.5 % Saponin/ 1 % BSA/PBS supplemented with 1 µl FcBlock antibody and

incubated for 20 min. Then 1 µl of Flag-FITC antibody was added to each sample. After 45 min incubation, cells were washed three times with 3 ml PBS prior to flow cytometry analysis.

6.3.2 Intracellular protein staining in COS-7 cells for fluorescence microscopy

For detection of the intracellular localization of proteins COS-7 cells growing on cover slips were transfected as described above (see 6.2.3). After 24 h cells were washed once with 300 µl cold PBS (per 24 well) and then fixed and permeabilized by incubation in 200 µl cold methanol (-20 °C). After 4 rounds of washing with 300 µl 0.1 % BSA /PBS, blocking was performed through addition of 10 % donkey serum/PBS for 30 min at room temperature. The cells were then washed once with 0.1 % BSA/PBS before 200 µl of a primary antibody solution in 0.1 %BSA/PBS (1:2000 for anti-Flag M5 and 1:200 for anti-myc 9E10) was added. After incubation for 1 to 2 h at room temperature, the antibody solution was removed and the cells washed four times with 300 µl 0.1 % BSA/PBS. Subsequently, secondary antibodies were added (anti-mouse Cy3 or anti-mouse Alexa-488) at a dilution of 1:400 in 0.1 % BSA/PBS and incubated for 1h at room temperature in the dark. Cells were washed once with 0.1 % BSA/PBS and then twice with DAPI (1:8000) in 0.1 % BSA/PBS (5 min incubation time). DAPI is a fluorescent dye that strongly binds to DNA, therefore leading to blue fluorescing cell nuclei. After two final washes, the cover slips were mounted in Mowiol on slides for fluorescence microscopy using an Axioplan 2 microscope (Zeiss).

6.4 Biochemical methods

6.4.1 Determination of protein concentrations

For colorimetric determination of protein concentrations a Protein dye reagent concentrate (Biorad) was used according to manufacturer's instructions. Absorption at 595 nm was measured and compared to BSA standard values.

6.4.2 Sodium dodecylsulfate polyacrylamide-gel electrophoresis (SDS-PAGE)

Proteins were separated by discontinuous SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Gels were produced by co-polymerization of acrylamide with the crosslinker N,N'-methylenebisacrylamide in the presence of the radical starter ammonium peroxodisulfate (APS) and the catalyst tetramethylethylenediamine (TEMED). The pore size of gels was regulated by varying the concentration of the acrylamid mix (see chapter 5.9 for

detailed protocols). Proteins are focused in a stacking gel with wide pores at pH 6.8 before entering the separation gel. Prior to SDS PAGE, samples were mixed with 6x SDS-sample buffer and boiled for 3 min. To estimate the molecular weight of separated proteins pre-stained marker proteins of known size were loaded. Electrophoresis was performed in SDS electrophoresis buffer at 75 V (stacking gel) for 30 min and 60 - 90 min at 150 V (separation gel).

6.4.3 Western Blotting

Proteins that were separated by SDS-PAGE were transferred onto PVDF-membranes (Western Blotting) for immunodetection. PVDF membranes have to be activated by soaking in methanol. SDS gel and activated membrane were placed between filter paper, which was soaked in blotting buffer, in a semi-dry blotting apparatus. Electrotransfer of the proteins was performed at 60 mA per gel for 60 – 80 min.

6.4.4 Immunodetection

For immunodetection PVDF membranes were first blocked in 3 % BSA/PBS-T for at least 60 min to saturate unspecific binding sites and then incubated with the protein-specific first antibody solution (antibody 1:1000 – 1:5000 in 1.5 % BSA/PBS-T) overnight at 4 °C. The membranes were washed 4 times for at least 5 min in PBS-T before adding the horseradish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:10000 in 1.5 % BSA/PBS-T. The secondary antibodies were incubated with the membranes for 60 to 120 min. Afterwards membranes were washed 4 times for 10 min with PBS-T and once shortly with water. For visualizing proteins by enhanced chemiluminescence the membranes were incubated for 2 min in a mix of 10 ml solution A, 1 ml solution B and 3 µl 35% H_2O_2. The reaction of HRP with its substrate leads to chemiluminescent radiation, which can be detected by X-ray films, which were exposed to the membranes for different time periods. To detect another protein on the same membrane, bound antibodies were stripped off either by incubation in stripping buffer A at room temperature overnight or in stripping buffer B for 30 min at 65 °C in a water bath with shaker. After thorough washing with PBS-T membranes can be blocked again for a second round of protein immunodetection.

6.4.5 (Co-)immunoprecipitations

In immunoprecipitation experiments (IPs) proteins are bound by specific antibodies and the protein/antibody-complex is precipitated from cell lysates by Protein G or Protein A sepharose (PGS or PAS), which bind to the constant regions of IgG, IgM and IgA antibody

subclasses and can be pelleted by centrifugation. If buffer conditions are suitable, proteins binding to the protein of interest are co-precipitated and can be detected on Western Blots (CoIP). In that way protein-protein interactions can be investigated.

For all CoIPs, HEK293 or Jurkat T cells were washed once with cold PBS and then lysed in CoIP buffer. For HEK293 cells CoIP buffer was directly added to the cell culture dish and the cell lysate was collected by scraping off the liquid and remaining cell debris. All lysates were incubated at 4 °C for 15 min in an overhead rotator and cleared by centrifugation at 14000 rpm for 10 min. 15 µl of the lysates were mixed with 6x SDS sample buffer for direct analysis by Western Blot (input or lysate aliquots). 1 - 2 µg primary antibody was added to the remaining lysate and incubated overnight rotating at 4 °C. Then, 15 µl of a 1:2 PGS suspension was added and incubated for another 1 - 2 hours at 4 °C. Finally, sepharose was pelleted by centrifugation (1500 rpm, 1 min), washed 3 times with 1 ml cold CoIP buffer, sucked dry with a flat pipett tip and taken up in 12 µl 2 x SDS sample buffer.

6.4.6 Detection of protein ubiquitination

For the detection of ubiquitination of a protein it is important that only the protein of interest and no binding partners are precipitated in IPs. This ensures that one specifically examines modification of the respective protein. For that purpose cells were lysed in ubiquitination lysis buffer, which contained 1 % SDS. Control CoIP experiments showed that lysis in this buffer abrogated protein-protein interactions of endogenous or overexpressed proteins (see Results Fig. 2.6).

For detection of ubiquitin-conjugation to Malt1 one 10 cm dish of HEK293 cells or 2 x 10^7 Jurkat T cells were washed once in cold PBS and then lysed in a maximal volume of 0.5 ml ubiquitination lysis buffer. Because lysis of nuclei occurs in this buffer, the cell lysates are highly viscous due to DNA denaturation. DNA was sheared by repeated passing through a capillary (26G) until viscosity decreased. Afterwards the lysates were cleared by centrifugation (14000 rpm, 5 min), diluted 10-fold with CoIP buffer and subjected to Malt1 immunoprecipitation (Malt1 H300 antibody).

6.4.7 Size-exclusion chromatography

The column material for size-exclusion chromatography, also called gelfiltration, is composed of inert polymer beads with a defined pore diameter. Proteins are separated according to their size and shape (apparent molecular weight): smaller molecules are able to enter the pores and therefore have a longer way to travel though the column. Thus, the smaller the protein, the later it will be eluted from the column. The void volume is characteristic for every column

type and represents the minimal volume a very large particle needs to pass the column without entering any pore. The elution volumes of globular proteins show a linear relation to the logarithm of their molecular weight. Therefore, gelfiltration columns can be calibrated by determining the elution volume of standard proteins to estimate the apparent molecular weight of a protein or protein complex of interest.

For analysis of CBM complex formation by gelfiltration approximately 1 x 10^8 Jurkat cells were stimulated with PMA/ionomycin for 15 min and then lysed in 1.3 ml CoIP buffer without glycerol. Lysates were cleared by two centrifugation steps (14000 rpm, 15 min and 45000 rpm, 30 min). Samples were loaded on a superose 6 column run at a flow rate of 0.3 ml/min. 0.5 ml fractions were collected. The gelfiltration runs and calibration of the column were performed by Rudolf Dettmer. For some experiments collected fractions were pooled before being subjected to immunoprecipitation with the indicated antibodies. For detection of Malt1 ubiquitination after gelfiltration, pooled fractions were supplemented with SDS to a final concentration of 1 % to disrupt protein-protein interactions, incubated for 30 min and then diluted 10-fold for immunoprecipitation.

6.5 Pull-down experiments

For pull-down experiments recombinantly expressed proteins bound to an affinity matrix are incubated with cell lysates. Binding partners will associate with the recombinant protein and can be detected by Western Blotting.

6.5.1 Recombinant protein expression and purification

IKKγ WT and the point mutants L329P and Y308S were expressed as fusion proteins with an N-terminal Strep-tag in *E. coli* BL21(DE3)pLysS. The 8 amino acid long Strep-tag (WSHPQFEK) was designed to fit the biotin-binding pocket of Strep-tactin, a modified form of streptavidin. A 5 ml overnight starter culture of transformed BL21 containing ampicillin was grown at 37 °C, used to inoculate 500 ml LB Amp the next day and grown further until an OD_{550nm} of 0.5 to 0.6 was reached. Recombinant protein expression was induced with 200 ng/ml anhydrotetracycline (AHT) and carried on for 12 to 14 h at 24 °C. Bacteria were harvested and lysed in 8 ml StrepIKKγ lysis buffer by sonication on ice (6 x 20 sec; intensity 85%, pulse 0.8). The lysates were cleared by centrifuging twice at 14000 rpm for 20 min and incubated with 100 µl Strep-Tactin resin for 4 h. Strep-Tactin beads were washed 4 times with 10 ml StrepIKKγ lysis buffer and once with 10 ml 0.1 % Triton buffer. Finally beads were

resuspended in 100 µl 0.1 % Triton buffer to yield a 1:1 bead suspension, that was used for pull-down experiments.

6.5.2 StrepIKKγ Pull-down

For each pull-down sample, 4 x 10^7 Jurkat T cells were left untreated or stimulated with PMA/ionomycin for 20 min. Jurkat T cells were lysed in 500 µl 1 % Triton lysis buffer. After centrifugation, lysates were diluted 10-fold with Triton dilution buffer and incubated with StrepIKKγ WT or mutant proteins bound to Strep-Tactin resin (40µl of 1:1 suspension) in an overhead rotator at 4 °C overnight. Beads were then washed 4 times with 10 ml 0.1 % Triton buffer, mixed with 12 µl 2x SDS sample buffer and subjected to Western Blot analysis.

6.6 In vitro ubiquitination assays

6.6.1 Recombinant protein expression and purification

For *in vitro* ubiquitination assays GST Malt1 482-813 WT, 6R and 11R and GST TRAF6 were recombinantly expressed in *E. coli* BL21 RIL. Bacteria were transformed and plated on LB ampicillin plates. 50 ml overnight starter cultures containing ampicillin were inoculated from a single colony and grown at 37 °C. The next day a glycerol stock was prepared by adding 250 µl of 87 % glycerol to 800 µl bacterial suspension for long term storage and inoculation of starter cultures for further protein expression rounds. The remaining culture was diluted 10-fold with ampicillin containing LB medium, grown at 30 °C to an OD_{600nm} of 0.6 to 0.7 and induced with 1 mM IPTG (final concentration). After 6 h of protein expression, bacteria were harvested and lysed in 6 ml *E. coli* lysis buffer by sonication (intensity 85 %, pulse 0.8) on ice for 6 x 20 sec. Lysates were cleared by 2 rounds of centrifugation in Eppendorf tubes at 14000 rpm for 15 min. 100 µl glutathione sepharose was incubated with the lysates for 3 - 4 h at 4 °C, then washed 4 times with 10 ml *E. coli* lysis buffer and once with buffer P4. Afterwards beads carrying GST Malt1 WT, 6R and 11R were resuspended in 100 µl buffer P4 to be used for *in vitro* ubiquitination assays. GST TRAF6 was eluted from the beads with GST elution buffer in three 200 µl steps. Protein amounts and purities were determined by SDS PAGE and Imperial protein staining (modified colloidal Coomassie staining, Pierce).

6.6.2 Ubiquitination reactions

In vitro ubiquitination reactions with a total volume of 20 µl were set up in buffer P1 according to Table 6.10 and incubated with slow shaking at 30 °C for 2 h. Afterwards reactions were diluted 10-fold with Ubiquitination buffer (1 % SDS) and heated for 5 min at 95 °C to elute GST Malt1 and mutants from the beads and to disrupt protein-protein interactions. After centrifugation the solution was separated from the Glutathione sepharose beads and diluted 10-fold with CoIP buffer. Malt1 was precipitated according to the standard immunoprecipitation protocol for ubiquitination (see 6.4.6) and precipitates were subjected to Western Blot analysis.

	amount
GST Malt1 WT, 6R or 11R	7 – 10 µl of bead suspension
GST TRAF6	10 µl
Ubiquitin activating enzyme E1	50 nM
UbcH13/Uev1a complex (E2)	875 nM
Ubiquitin WT, K63R and K48R	150 µM
10x Energy regenerating solution (contains MgCl2, ATP and ATP regenerating enzymes)	1x

Table 6.10: Protocol for *in vitro* ubiquitination reactions using GST Malt1 and GST TRAF6

6.7 NF-κB reporter assay

To determine NF-κB activation by overexpression of signaling mediators, HEK293 cells were transfected as described above (see 6.2.1). In addition to 1 - 3 µg of the plasmid DNA of interest, 5 ng 6x NF-κB reporter plasmid (for inducible expression of Firefly luciferase) and 400 ng TKluc plasmid (for constitutive expression of *Renilla* luciferase) were transfected per 6-well. 24 h after transfection, cells were washed once with cold PBS and lysed in 250 µl Passive lysis buffer (Promega). Lysis was carried out by shaking plates for 15 to 30 min at room temperature. 5 µl of these lysates was analyzed using the Dual-Glo Luciferase™ Assay system according to manufacturer's instructions with a microplate reader. The Dual-Glo Luciferase reagent, which acts as substrate for Firefly luciferase, was added first and the luminescence signal was measured. Addition of Dual-Glo Stop & Glo solution quenched the luminescence from the Firefly reaction and supplied the substrate for *Renilla* luciferase. Duplicates of each transfection were measured. The luminescence signal of the constitutively expressed *Renilla* luciferase is used as an internal control. For analysis, the ratio of Firefly and *Renilla* signals was calculated for each sample of three independent experiments.